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Analogues of luteinizing hormone-releasing hormone containing cytotoxic groups

(targeted chemotherapeutic agents/alkylating agents/methotrexate/doxorubicin/receptor binding)

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ABSTRACT **In an attempt to produce better cytotoxic analogues, chemotherapeutic antineoplastic radicals including an alkylating nitrogen mustard derivative of D-phenylalanine (D-melphalan), reactive cyclopropane, anthraquinone derivatives [2-(hydroxymethyl)anthraquinone and the anticancer antibiotic doxorubicin], and an antimetabolite (methotrexate) were coupled to suitably modified agonists and antagonists of luteinizing hormone-releasing hormone (LH-RH). Analogues with D-lysine^6 and D-ornithine^6 or N^{ϵ}-(2,3-diaminopropionyl)-D-lysine and N^{δ}-(2,3-diaminopropionyl)-D-ornithine were used as carriers for one or two cytotoxic moieties. The enhanced biological activities produced by the incorporation of D amino acids into position 6 of the agonistic analogues were further increased by the attachment of hydrophobic cytotoxic groups, resulting in compounds with 10–50 times higher activity than LH-RH. Most of the monosubstituted agonistic analogues showed high affinities for the membrane receptors of human breast cancer cells, while the receptor binding affinities of peptides containing two cytotoxic side chains were lower. Antagonistic carriers [Ac-D-Nal(2)^1,D-Phe(4Cl)^2,D-Trp^3,Arg^5,D-Lys^6,D-Ala^{10}]LH-RH [where Nal(2) is 3-(2-naphthyl)alanine], [Ac-D-Nal(2)^1,D-Phe(4Cl)^2,D-Trp^3,Arg^5,N^{ϵ}-(2,3-diaminopropionyl)-D-Lys^6,D-Ala^{10}]LH-RH, and their D-Pal(3)^3 homologs [Pal(3) is 3-(3-pyridyl)alanine] as well as [Ac-D-Nal(2)^1,D-Phe(4Cl)^2,D-Pal(3)^3,Tyr^5,N^{ϵ}-(2,3-diaminopropionyl)-D-Lys^6,D-Ala^{10}]LH-RH were linked to cytotoxic compounds. The hybrid molecules inhibited ovulation in rats at doses of 10 μg and suppressed LH release *in vitro*. The receptor binding of cytotoxic analogues was decreased compared to the precursor peptides, although analogues with 2-(hydroxymethyl)anthraquinone hemiglutarate had high affinities. All of the cytotoxic analogues tested inhibited [^{3}H]thymidine incorporation into DNA in cultures of human breast and prostate cancer cell lines. Some cytotoxic analogues also significantly suppressed the growth of mammary and prostate cancers *in vivo* in animal models.**

Chemotherapy has been, for many decades, one of the main approaches for the treatment of malignant neoplasms. Despite the development of modern, more specific cytotoxic drugs, their nonselective action on cells other than cancerous ones remains a major problem. A recent modality for the treatment of hormone-sensitive tumors is based on the use of agonists and antagonists of luteinizing hormone-releasing hormone (LH-RH) (1). Some LH-RH agonists substituted in position 6, 10, or both are much more active than LH-RH and also possess prolonged activity (1–3). Changes in positions 1, 2, 3, and 6 and occasionally in positions 5 and 10 of the LH-RH molecule lead to the formation of powerful antagonists (1–4), which inhibit the release of LH and follicle-stimulating hormone from the pituitary, create a state of sex-steroid deprivation, and thus have potential therapeutic applications in the treatment of some hormone-dependent cancers such as those of prostate and breast (1, 5).

Ideal anticancer drugs would theoretically be those that eradicate cancer cells without harming normal cells. Some hormonal peptide analogues carrying antineoplastic agents could be used for endocrine therapy and at the same time for targeted chemotherapy of cancers that possess receptors for their peptide moieties on tumor cell membranes. Combination of LH-RH analogues with cytotoxic compound into a hormone–drug conjugate is a good example of this approach. Such a hybrid molecule could exert the antitumor effect of an LH-RH agonist or antagonist and, at the same time, its nonpeptidic cytotoxic moiety could act as a chemotherapeutic agent that might be targeted to the tumor cells by their peptide portion. These types of analogues could bind to LH-RH receptors and provide some site selectivity for the cytotoxic radical. On the basis of this concept proposed by one of us (A.V.S.), Bajusz *et al.* synthesized several analogues of LH-RH with D-melphalan {D-Mel; 4-[bis(2-chloroethyl)amino]phenylalanine} (6) or metallopeptides (7) containing platinum, copper, and nickel with cytotoxic activities *in vitro*. In this paper, we describe additional highly potent LH-RH analogues containing clinically used chemotherapeutic agents that inhibit various phases of protein and nucleic acid biosynthesis. The radicals that were incorporated included alkylating D-Mel, cyclopropane, anthraquinone derivatives [such as the anticancer antibiotic doxorubicin (Dox)] and the antimetabolite methotrexate (MTX).

MATERIALS AND METHODS

Amino Acid Derivatives. Boc-D-Ala, Boc-Arg(Tos), Boc-Gly, Boc-His(Tos), Boc-Leu, Boc-D-Lys(2-ClZ), Boc-D-Orn(Z), Boc-D-Nal(2), Boc-D-Phe(*p*Cl), Boc-Pro, Boc-Ser(Bzl), Boc-D-Trp, Boc-Tyr(Cl_2Bzl), and pyroglutamic acid (Glp) were purchased from Bachem [Boc, *tert*-butoxycarbonyl; Tos, *p*-toluenesulfonyl; Z, benzyloxycarbonyl; Nal(2), 3-(2-naphthyl)alanine; Phe(*p*Cl), *p*-chlorophenylalanine; Cl_2Bzl, 2,6-dichlorobenzyl]. Boc-D-Pal(3) [Boc-protected 3-(3-pyridyl)-D-alanine] was kindly donated by

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Abbreviations: LH, luteinizing hormone; LH-RH, LH-releasing hormone; Nal(2), 3-(2-naphthyl)alanine; Pal(3), 3-(3-pyridyl)alanine; Mel, 4-[bis(2-chloroethyl)amino]phenylalanine; A_2pr, 2,3-diaminopropionic acid; Boc, *tert*-butoxycarbonyl; Pcp, pentachlorophenyl; MTX, methotrexate (4-amino-N^{10}-methylpteroyl-L-glutamic acid); Dox, doxorubicin; CPC, cyclopropanecarbonyl; AG, agonist; ANT, antagonist; HMAQG, 2-(hydroxymethyl)anthraquinone hemiglutarate; Glp, pyroglutamic acid; DMF, dimethylformamide.

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ASTA-PHARMA (Frankfurt). Boc_2A_2pr was prepared from L-2,3-diaminopropionic acid (A_2pr) (Calbiochem–Behring). Reaction of D-Mel first with di*tert*-butyldicarbonate and then with pentachlorophenyl (Pcp) trichloroacetate resulted in Boc-D-Mel-OPcp (6).

Cytotoxic Compounds. 2-(Hydroxymethyl)anthraquinone, Dox, MTX, and cyclopropanecarbonyl (CPC) chloride were purchased from Aldrich. 2-(Hydroxymethyl)anthraquinone hemiglutarate (HMAQG) was prepared by refluxing 2-(hydroxymethyl)anthraquinone with glutaric acid anhydride in anhydrous pyridine for 24 hr and was purified by recrystallization from ethyl acetate/hexane (1:1, vol/vol).

Precursor Peptides with D-Lysine or D-Ornithine Residue. Agonistic and antagonistic LH-RH analogues containing D-Lys^6 or D-Orn^6 were prepared by standard solid-phase peptide synthesis, as described (6). Acylation of these peptides with Boc_2A_2pr followed by deprotection with trifluoroacetic acid resulted in *N*-diaminopropionyl-D-Lys^6 (or D-Orn^6) derivatives. [(N^{ε}-Glutaryl-D-Lys)^6]LH-RH was prepared by acylation of [D-Lys^6]LH-RH with glutaric acid anhydride in the presence of triethylamine in dimethylformamide (DMF).

Peptides with Cytotoxic Moiety. LH-RH analogues containing D-Mel were prepared by reacting the free amino group(s) in the side chain of precursor peptides with Boc-D-Mel-OPcp followed by the removal of the Boc group. The same starting peptides were acylated with CPC chloride to obtain [(CPC-D-Lys)^6]-, [(CPC-D-Orn)^6]-, [(CPC_2-A_2pr-D-Lys)^6], and [(CPC_2-A_2pr-D-Orn)^6]LH-RH analogues.

Preparation of MTX-containing peptides was performed by acylation of the above-mentioned LH-RH analogues with MTX. The glutamic acid residue of MTX was activated with diisopropylcarbodiimide in DMF and was reacted at 0°C for 10 hr with [D-Lys^6] or [D-Orn^6] analogues of LH-RH. Incorporation of an anthraquinone derivative into a peptide was carried out by preparing the HOBt ester of HMAQG *in situ* and reacting it with the corresponding precursor peptides at 0°C for 24 hr. The synthesis of Glp-His-Trp-Ser-Tyr-D-Lys(glutaryl-Dox)-Leu-Arg-Pro-Gly-NH_2 was performed by coupling the amino sugar moiety of Dox to the glutaryl side chain of the parent peptide. The HOBt ester of the glutaryl side chain was prepared *in situ* in DMF by addition of diisopropylcarbodiimide and was reacted with Dox at 0°C overnight.

HPLC. All synthetic peptides were purified as described (4, 6, 7). Amino acid analyses were also performed as reported (4, 6, 7).

LH-Releasing and LH-RH-Inhibiting Activities. Activities were evaluated *in vitro* by using a superfused rat pituitary cell system (8, 9). *In vivo* antiovulatory activity of peptides was determined in 4-day-cycling rats as described (10).

Receptor Binding. The affinity of peptides to human breast cancer cell membranes was determined by using ^{125}I-labeled [D-Trp^6]LH-RH (11).

Cytotoxicity Test. The ability of peptides to inhibit incorporation of [^{3}H]thymidine into DNA of monolayer cultures of the human breast and prostate cancer cell lines was assayed as described (12, 13).

RESULTS

Preparation of LH-RH Analogues with Cytotoxic Moieties. Precursor peptides of cytotoxic analogues were synthesized by incorporation of D-Lys or D-Orn into position 6 of the native LH-RH sequence (AG-1 and AG-2) and by incorporation of D-Lys into three antagonistic analogues (ANT-1, ANT-2, and ANT-3) (AG, agonist; ANT, antagonist). To obtain precursors for incorporation of two cytotoxic groups, these peptides were acylated with 2,3-diaminopropionic acid, resulting in D-Lys(A_2pr)- and D-Orn(A_2pr)-containing precursors (AG-3, AG-4, ANT-4, ANT-5, and ANT-6). Accordingly, the general formulae are as follows:

AG-1, AG-2 Glp-His-Trp-Ser-Tyr-R^6-Leu-Arg-Pro-Gly-NH_2;

AG-3, AG-4 Glp-His-Trp-Ser-Tyr-R^6(A_2pr)-Leu-Arg-Pro-Gly-NH_2;

ANT-1, ANT-2 Ac-D-Nal(2)-D-Phe(4Cl)-R^3-Ser-Arg-D-Lys-Leu-Arg-Pro-D-Ala-NH_2;

ANT-3, Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Lys-Leu-Arg-Pro-D-Ala-NH_2;

ANT-4, ANT-5, Ac-D-Nal(2)-D-Phe(4Cl)-R^3-Ser-Arg-D-Lys-(A_2pr)-Leu-Arg-Pro-D-Ala-NH_2;

ANT-6, Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Lys-(A_2pr)-Leu-Arg-Pro-D-Ala-NH_2;

where R^3 is D-Trp or D-Pal(3) and R^6 is D-Lys or D-Orn.

The two isomers of MTX conjugates, formed by the indiscriminate activation of the α and γ carboxyl groups of the glutamic acid moiety in MTX, were separated only in the case of AG-1 (VIII), although the two structural isomers formed with other precursors also have different retention times (XV, XXII, and XXIX).

Agonistic and Antagonistic Properties. LH-releasing activity of agonistic derivatives (I–XV) and their precursor peptides in a dispersed rat pituitary superfusion system is shown in Table 1. Cytotoxic analogues showed 10–56 times higher potency than LH-RH itself and were 2–8 times more potent in releasing LH than their precursor peptides. LH-RH inhibiting potencies of the antagonistic conjugates (XVI–XXX) and their parent peptides are presented in Table 2. Antago-

Table 1. Substituents, LH-releasing activity, and receptor binding to human breast cancer membranes of agonist-type analogues based on Glp-His-Trp-Ser-Tyr-D-R^6(AX)-Leu-Arg-Pro-Gly-NH_2 peptides containing cytotoxic moieties and their parent peptides

Peptide					Affinity constant*	
	Code no.	A†	X†	Relative activity‡	K_{a1}, nM^{-1}	K_{a2}, μM^{-1}
Peptides with D-Lys^6						
AG-1	—	—	—	7	5.88	4.21
I	AJ-23	—	D-Mel		6.74	1.07
II	AJ-11	A_2pr	D-Mel_2		30.48	3.45
III	T-108	—	CPC	52	1.56	—
IV	T-111	A_2pr	CPC_2	25	0.14	—
V	T-98	—	HMAQG	35	4.0	5.26
VI	T-119	A_2pr	$HMAQG_2$	30	NB	NB
VII	T-107	—	DoxG	12	—	14.4
VIII	AJ-04	—	MTX	10	5.42	1.59
Peptides with D-Orn^6						
AG-2		—	—			
IX	AJ-24	—	D-Mel		11.51	0.34
X	AJ-25	A_2pr	D-Mel_2		6.47	—
XI	T-113	—	CPC	40	—	44.2
XII	T-135	A_2pr	CPC_2		NB	NB
XIII	T-118	—	HMAQG	56	—	1.3
XIV	T-133	A_2pr	$HMAQG_2$	26		
IX	AJ-15	—	MTX	11		

NB, no binding.

*Affinity constants of the peptides to human breast cancer membrane receptors were determined by using ^{125}I-labeled [D-Trp^6]LH-RH.

†A and X are substituents on the R^6 side chain (D-Lys or D-Orn).

‡LH-releasing activity was determined in a perfused rat pituitary system and is expressed relative to LH-RH decapeptide = 1.0.

nists containing D-Pal(3)^3 (XVI–XXII) were more active than peptides with a D-Trp^3 substitution (XXIII–XXIX). Condensation of precursor D-Pal(3)^3 antagonists with cytotoxic compounds resulted in some peptides with extremely high and long-lasting inhibitory effects (XVI, XVIII, XX).

Antiovulatory activity *in vivo* of D-Pal(3)^3 analogues proved to be high, comparable to that of their precursors, whereas D-Trp^3-containing analogues had moderate antagonistic activities (Table 2).

Receptor Binding Affinity. Binding of cytotoxic peptides to human breast cancer membranes was determined by using ^{125}I-labeled [D-Trp^6]LH-RH as ligand (Tables 1 and 2). Agonists with the same cytotoxic substituents based on [D-Orn^6]LH-RH instead of [D-Lys^6]LH-RH showed lower binding or no binding. Incorporation of D-Mel into agonists resulted in peptides with high binding affinity (I, II). Affinities of the antagonistic analogues containing cytotoxic groups proved to be lower compared to their precursor peptides, although XX, XXIV, XXVII, XXVIII, and XXX showed high binding.

Cytotoxicity. Inhibition by our analogues of [^{3}H]thymidine incorporation was evaluated in cell cultures of SKBr-3, MDA-MB-231, T-47D, and MCF-7 human breast cancer lines as well as PC-3 and LNCaP prostate cancer cell lines. All of the compounds tested reduced [^{3}H]thymidine incorporation (Table 3) but the response varied in different cell lines—e.g., VIII exerted good inhibition on the LNCaP line but was almost ineffective on the PC-3 line. Compounds VII, VIII, and XXVI were more cytotoxic after incubation for 4 hr than after a 24-hr incubation time, but the cytotoxicity was cell line dependent. Some peptides with two D-Mel showed high cytotoxicity on SKBr-3 and MDA-MB-231 lines (II) and on PC-3 lines (XVII). Incorporation of two anthraquinone molecules into antagonistic LH-RH analogues gave peptides with extremely high inhibitory potency on almost all cell lines.

DISCUSSION

Several approaches are being tried to increase the selectivity of the cytotoxic action of different antineoplastic drugs. Drug targeting is one of these attempts: cytotoxic groups can be attached to an appropriate carrier molecule in order to be transported to the tumor cells in a targeted manner.

Many human tumors are hormone dependent or hormone responsive and contain hormone receptors (1, 5, 11, 14–19). Receptors for peptide hormones such as LH-RH, somatostatin, and bombesin and growth factors such as epidermal growth factor and insulin-like growth factor I have been detected in cancers of the prostate, breast, pancreas, ovary, endometrium, and colon as well as in brain tumors (1, 5, 11, 14, 19). It has been shown by us and other investigators that both agonistic and antagonistic analogues of LH-RH bind to human breast cancer cell membranes (11, 18). Agonistic and antagonistic analogues of LH-RH were also reported to be internalized in pituitary cells by endocytosis (20).

Due to the heterogeneity of solid tumors, only the growth of sex hormone-dependent cells is inhibited by sex steroid deprivation therapy (18, 21), but hormone-insensitive cells are able to proliferate and eventually become predominant (21). However, in human breast cancer specimens, there is no statistically significant correlation between receptor binding of estrogen and [D-Trp^6]LH-RH (18). Among 92 estrogen receptor-negative specimens, 47 showed binding sites for [D-Trp^6]LH-RH (18). This suggests that LH-RH analogues could be considered for the treatment of such tumors. Similar considerations could be extended to prostate cancer patients (16, 21). A combination of hormonal manipulation with targeted chemotherapy would enhance the efficacy of the treatment. This work and previous studies (5–7) show that LH-RH analogues might serve as carriers for chemotherapeutic agents. The conjugates would bind to receptors on cell membranes of tumors. This binding could be followed by internalization and a chain of events that might result in interference with the replication of neoplastic cells or even their destruction by the derivative of the cytotoxic analogue. The release of the cytotoxic moieties or their biologically active derivatives from the carrier hormone or the splitting of the bond between the drug and the peptide may not be an

Table 2. Substituents, antiovulatory activity, LH-inhibition *in vitro*, and receptor binding of antagonist-type analogues based on Ac-D-Nal(2)-D-Phe(*p*Cl)-*R*^{3}-Ser-*R*^{5}-D-Lys(AX)-Leu-Arg-Pro-D-Ala-NH_2 peptides containing cytotoxic moieties and their precursor peptides

Peptide				% blockade of ovulation*	Dose, nM	% inhibition of LH response			Affinity constant†	
	Code no.	A‡	X‡			0 min	30 min	60 min	K_{a1}, nM^{-1}	K_{a2}, μM^{-1}
				Peptides with D-Pal^3 and Arg^5						
ANT-1		—	—		3	78	44	40	2.16	—
XVI	AJ-10	—	D-Mel	100	3	80	71	62	0.97	1.34
XVII	AJ-26	A_2pr	D-Mel_2		3	29	40	43	NB	NB
XVIII	T-116	—	CPC	100	1	75	58	50	NB	NB
XIX	T-125	A_2pr	CPC_2	100	1	60	43	32	1.52	—
XX	T-117	—	HMAQG	100	1	50	50	50	3.22	0.3
XXI	T-122	A_2pr	$HMAQG_2$	40	1	43	43	35	—	17.8
XXII	AJ-06	—	MTX	100	3	86	33	23	1.9	0.3
				Peptides with D-Trp^3 and Arg^5						
ANT-2		—	—		3	45	14	8	0.65	—
XXIII	AJ-09	—	D-Mel	40	3	39	22	20	1.48	5.1
XXIV	AJ-30	A_2pr	D-Mel_2	20	3	36	35	35	3.82	—
XXV	T-123	—	CPC	80	1	50	25	10	NB	NB
XXVI	T-124	A_2pr	CPC_2	60	1	45	10	0	0.42	—
XXVII	T-120	—	HMAQG		1	27	20	15	10.2	3.7
XXVIII	T-121	A_2pr	$HMAQG_2$		3	17	20	18	83.3	28.5
XXIX	AJ-29	—	MTX	60	3	40	10	10	NB	—
				Peptide with D-Pal(3)^3 and Tyr^5						
XXX	T-144	A_2pr	$HMAQG_2$		3	0	4	14	8.33	6.66

Peptides were perfused through rat pituitary cells for 12 min at 1–3 nM. During the last 3 min, 3 nM LH-RH was also given (0-min response). LH-RH (3 nM) was also administered 30 and 60 min later for 3 min. NB, no binding.

*Based on the number of rats that did not ovulate/number of animals tested (n = 4–8) at a dose of 10 μg per rat.

†Affinity constants of the peptides to human breast cancer membrane receptors.

‡A and X are substituents on the D-Lys side chain.

Table 3. Inhibition of [^{3}H]thymidine incorporation into DNA by LH-RH agonists and antagonists with cytotoxic moieties in human breast cancer and prostate cancer cell lines

Peptide				% inhibition of [^{3}H]thymidine incorporation											
				SKBr-3†		MDA-MB-231†		T-47D†		MCF-7†		PC-3‡		LNCaP‡	
	Code no.	X*	Dose, μg	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr
II	AJ-11	D-Mel$_2$	1		30	20	15	31	15						
			10		66	20	62	39	28						
V	T-98	HMAQG	1												
			10								71		51		
VI	T-119	HMAQG$_2$	1								24				
			10								44				
VII	T-107	DoxG	1	27	14	20		32	11	19	0				
			10	24	0	9		44	10	34	61		6		
VIII	AJ-04	MTX	1	21	16	31	0	38	26					24	39
			10	36	10	23	8	54	41				14	20	76
XVII	AJ-26	D-Mel$_2$	1										30		
			10										76		
XXVI	T-124	CPC$_2$	1	37	18	25	8	44	22	25	8				
			10	42	29	73	11	50	12	34	37				
XXVIII	T-121	HMAQG$_2$	1	30	9	36		37	20		30			48	59
			10	53	88	40	90	41	54		60		59	39	70
XXX	T-144	HMAQG$_2$									47				
											71				

*Cytotoxic radical.
†Human breast cancer lines.
‡Human prostate cancer lines.

essential requirement, since drugs linked to peptides or proteins by nonhydrolyzable covalent bonds can produce active drug–carrier conjugates as described by Varga (22).

In the present study, anticancer drugs or structurally related cytotoxic radicals were coupled to LH-RH agonists (AG-1 and -2) and antagonists (ANT-1 and -2). By preparation of analogues containing two amino groups (AG-3 and -4; ANT-4, -5, and -6), we were able to double the cytotoxic moiety/peptide ratio.

Hydrophobic D amino acid residues at position 6 of LH-RH greatly increase the LH-releasing activity of the parent hormones (3, 6). Substitution of [D-Lys^6]- or [D-Orn^6]LH-RH with their hydrophobic cyclopropane (III, XI) or anthraquinone derivatives (V, XIII) led to highly active conjugates. Such residues are also favorable in antagonistic analogues (XVIII, XX), provided they are paired with the replacement of the neighboring Tyr^5 by the hydrophilic Arg and proper substitution in the N-terminal tripeptide fragment. D-Trp in position 3 (XXIII–XXIX) was less favorable than D-Pal(3) (XVI–XXII) with respect to either their antiovulatory or antagonistic activity (Table 2). One analog, T-144 (XXX), having Pal(3)^3 and Tyr^5 in the peptide chain showed very high cytotoxic activity *in vitro*.

Alkylating agents used in the treatment of cancer have a basically nonselective mechanism of action (23). They act by exerting cytotoxic effects through transfer of their alkyl groups to various cell constituents. Alkylation of DNA within the nucleus probably represents the major interaction that leads to cell death. Nitrogen mustards (chlorambucil, cyclophosphamide, Mel) are among the oldest anticancer drugs in clinical use. Initially the incorporation of alkylating chlorambucil 4-[bis(2-chloroethyl)amino]benzenebutanoic acid into LH-RH agonists and antagonists in an attempt to make contraceptive analogues led to compounds with low or no activity (24). Our work shows that LH-RH analogues containing D-Mel have high agonistic and antagonistic activity and bind to the rat pituitary, human breast, and human prostate cancer cell membranes with high affinity (6). We could demonstrate significant cytotoxic activity of these compounds as based on inhibition of [^{3}H]thymidine incorporation in cultures of human breast cancer cell line T-47D and rat mammary tumor cell line MT-4 and MT-5 (6).

Hybrid molecules from AG-1, AG-3, and D-Mel (I, II) showed outstanding binding to breast cancer receptors. The lack of binding or the low-affinity constants of antagonists with D-Mel (XVI, XVII, XXIII) could be explained by the assumption (4) that the sets of subsites involved in the interaction of the LH-RH receptor with LH-RH agonists are not identical with those mediating the binding of LH-RH antagonists. Nevertheless, XXIV containing two D-Mel residues had remarkable affinity. Our earlier compounds (6) having D-Mel in position 6 in the peptide chain similarly did not alkylate the receptors.

Cyclopropane with its three-membered ring is another alkylating agent. Receptor binding of analogues with CPC (III, IV, XI, XIX, XXVI) to cancer cell membrane was decreased or completely absent (XII, XVIII, XXV). This indicates that no alkylation of receptors has occurred. At the same time, XVIII exhibited a strong and prolonged antagonistic effect *in vitro*.

Many drugs used in cancer chemotherapy contain a quinone group in their structure. Anthracycline antitumor antibiotics such as Dox bind to DNA through intercalation between specific bases and block the synthesis of new RNA or DNA (or both), cause DNA strand scission, and interfere with cell replication (25). When Dox was coupled to peptide carrier melanotropin, the conjugate proved to be more toxic to murine melanoma cells than the free drug (22). Coupling Dox to AG-1 through a glutaric acid bridge resulted in a conjugate (VII) with low receptor binding and moderately elevated agonistic activity.

2-(Hydroxymethyl)anthraquinone derivatives have cytotoxic activity on hypoxic neoplastic cells (26). Compounds V, VI, XIII, and XIV contain 2-(hydroxymethyl)anthraquinone substituent bridged to agonistic LH-RH analogues. Their high agonistic activity is probably due to the hydrophobic anthraquinone structure, although their receptor binding on cancer cell membranes is low. In contrast, antagonistic analogues (XX, XXVII, and XXX) bind to receptors with 2–10 times higher affinity than their parent peptides. The

extremely high and long-lasting antagonistic activity of XX (T-117) might be explained by its slow dissociation from the receptor binding sites.

Several antimetabolites are of potential chemotherapeutic interest because of their importance in cellular folate metabolism (27). MTX is a folic acid antagonist that inhibits the function of dihydrofolate reductase and in this way interrupts the synthesis of thymidilate, purine nucleotides, and the amino acids serine and methionine, thereby interfering with the formation of DNA, RNA, and proteins (27). MTX containing LH-RH agonist (VIII) had good binding affinity and cytotoxic activity.

Some of these LH-RH analogues were evaluated *in vitro* for cytotoxicity in cell cultures of various mammary and prostate cancer cell lines. Compounds I–XXX inhibited [^{3}H]thymidine incorporation, but the inhibition rates differed from cell line to cell line. Several factors influence the cytotoxicity of these compounds, including receptor binding, which was discussed above, but generally the antagonistic analogues carrying cytotoxic radicals bind with lower affinities than the agonistic ones. Pure agonistic analogues are internalized in pituitary cells much faster (1–3 min) than antagonistic peptides (20). In spite of this, there were no significant differences between cytotoxicity of I–XV and XVI–XXX. In addition to exerting cytotoxic activity *in vitro*, some analogues reported here inhibited the growth of Dunning R3327H prostate cancer in rats and breast cancers in mice. Cytotoxic LH-RH analogues (VIII), AJ-04 (agonist [D-Lys^6]LH-RH linked to MTX, (V) T-98 {[D-Lys^6]LH-RH coupled to HMAQG and (XXVIII) T-121 (antagonist containing 2 residues of HMAQG) produced a significant inhibition of tumor growth in female BDF_1 mice bearing MTX (3.2)/Ovex}, estrogen-independent mammary tumors. These results indicate that LH-RH analogues containing cytotoxic radicals retain their hormonal activity after administration *in vivo* and can apparently be bound to tumors that have receptors for LH-RH. Our studies suggest the merit of further development of LH-RH analogues containing cytotoxic radicals.

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